DENITRIFICATION IN SOIL

I. METHODS OF INVESTIGATION

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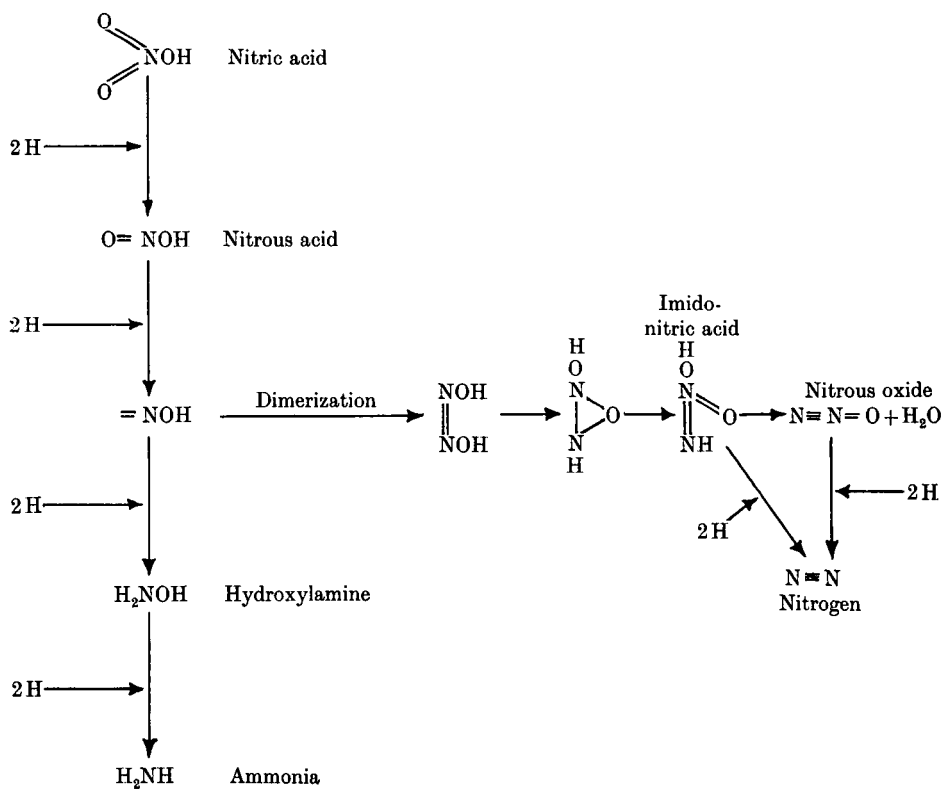
(With Six Text-figures)

The term denitrification is now reserved to describe the microbial process whereby nitrate is reduced to gaseous nitrogen compounds such as nitrous oxide and nitrogen. It has been used to describe other processes involving the disappearance of nitrate such as the assimilatory reduction of nitrate accomplished by higher plants and micro-organisms to meet their requirements for organic nitrogen, but denitrification differs from these in that it leads to gaseous loss of nitrogen.

All denitrifying bacteria so far studied have been found to be facultative anaerobes. Provided an oxidizable substrate is present in the medium, they can grow under anaerobic conditions in the presence of nitrate or under aerobic conditions in the presence of any suitable source of nitrogen. The oxidizable substrate can be inorganic as well as organic, e.g. autotrophic bacteria have been described which can grow anaerobically in the presence of nitrate at the expense of molecular hydrogen (Niklewski, 1914; Verhoeven, Koster & van Nievelt, 1954) or of sulphur, sulphide or thiosulphate (Nathansohn, 1902; Beijerinck, 1920; Baalsrud & Baalsrud, 1954). These observations are concordant with the theory, first proposed by Weissenberg (1897, 1902), that microbial denitrification is equivalent to aerobic oxidation with nitrate replacing oxygen as the ultimate oxidizing agent, and it is now accepted that, in the process of denitrification, nitrate instead of oxygen serves as the final hydrogen (or electron) acceptor in enzymic dehydrogenations of the organic or inorganic substrate. The mechanism of denitrification has not been fully elucidated; current concepts are expressed in the following scheme for dissimilatory nitrate reduction proposed by Kluyver & Verhoeven (1954):



The demonstration by Gayon & Dupetit (1886) that soil contains bacteria capable of reducing nitrate to molecular nitrogen and nitrous oxide led to much speculation concerning the loss of nitrogen from soil by denitrification and considerable alarm was caused in agricultural circles when Wagner (1895) published the results of an extensive investigation from which he concluded that serious loss of nitrogen may occur when nitrate is used as a fertilizer. This conclusion was soon challenged by others who pointed out that it is unusual to add organic matter and nitrate to soil at the same time as was done in Wagner's experiments, and it subsequently became generally accepted that loss of nitrogen by denitrification is negligible in well-aerated soils provided no addition or organic matter is made when nitrate is applied. Further work tended to confirm this view, and when Waksman (1927) reviewed work on denitrification his conclusion was that 'the phenomenon of denitrification is of no economic significance in well-aerated, not too moist soils, in the presence of moderate amounts of organic matter or nitrate'. This conclusion was not seriously questioned until Broadbent (1951) published the results of an investigation of denitrification in Californian soils which indicated that denitrification is of considerable economic significance in aerated soils in the presence of moderate amounts of organic matter and nitrate. For example, he found that when a sandy loam containing 1290 p.p.m. of total nitrogen and 64 p.p.m. of nitrate nitrogen was incubated in an aeration stream containing 21 % oxygen it lost 250 p.p.m. of nitrogen in 7 days. Subsequent work by Mann & Barnes (1951), Broadbent & Stojanovic (1952), Verhoeven (1952), Wijler & Delwiche (1954), Arnold (1954) and Wallace & Smith (1954) have lent support to the view that significant loss of nitrogen from soil may occur by the process of denitrification. Further support can be taken from soil nitrogen balance sheets obtained from lysimeter experiments (see Allison, 1955) as they frequently show substantial losses of nitrogen that are difficult to explain unless it is assumed that considerable loss of soil nitrogen occurs by gaseous evolution.

Although definite progress has been made in recent work on denitrification in soil by the use of the mass spectrometer and the infra-red technique for the identification and estimation of the gaseous products of denitrification it is still difficult to evaluate much of the work in this field. The literature contains numerous contradictions and the validity of the results presented in many publications cannot be assessed because insufficient information is provided regarding the methods of investigation employed. For example, several reports of investigations in which loss of nitrogen was determined by total nitrogen analysis give no details of the method of analysis employed and few reports of such investigations even mention the difficulty of finding a method of determining nitrogen that will include all the non-gaseous compounds of nitrogen likely to be present in soil during denitrification.

The study of denitrification in the field is beset with experimental difficulties that have so far proved insuperable and most of the information now available has been obtained from laboratory experiments in which denitrification has been studied either by estimating the gaseous products of denitrification or by following loss of nitrogen by total nitrogen analysis. To obtain accurate results by either of these methods without using labelled nitrate, it is necessary to add nitrate to soil in amounts considerably greater than those usually applied in the field. A further disadvantage of these methods when unlabelled nitrate is used is that if denitrification is accompanied by nitrogen fixation they will measure only the net loss of nitrogen resulting from these two processes. However, it appears unlikely that significant fixation of nitrogen will occur during the process of denitrification since various workers (e.g. Delwiche & Wijler, 1956) have reported that nitrogen fixation is suppressed by small concentrations of nitrate.

The object of the work reported in this series of papers was to obtain sufficiently detailed information regarding the factors affecting denitrification in soil to permit an assessment of the importance of loss of nitrogen by denitrification under field conditions.

It was realized at the outset that more valuable information could be obtained from investigations using labelled nitrate, but as the facilities at our disposal for work with ^{15}N were too limited to permit an extensive study of denitrification in soil it was decided to carry out most of the work using unlabelled nitrate. In view of the difficulties associated with the identification and determination of the gaseous products of denitrification in work with unlabelled nitrate, denitrification was followed by determining loss of nitrogen by total nitrogen analysis. The soils used included some from the Broadbalk continuous wheat plots at Rothamsted which appear to lose 60–70 % of their annual addition of nitrogen by leaching or by gaseous evolution (see Russell, 1950).

The present paper deals with methods of investigating denitrification in soil. The subsequent paper gives the results obtained in studies on the factors affecting this process using the techniques described here.

EXPERIMENTAL

Materials

Soils. Relevant analytical data concerning the soils used are given in Table 1. Samples 1–4 were taken from the Broadbalk continuous wheat plots at

Rothamsted where the soil is derived from Clay-with-Flints overlying Chalk. Sample 1 was from the plot which had received no manure or fertilizer since 1839 (plot 3), sample 2 from the plot receiving farmyard manure annually (plot 2B), sample 3 from the plot given annual additions of nitrogen, phosphorus and potassium (plot 13), and sample 4 from a plot receiving complete minerals and nitrogen annually (plot 7). Sample 5 was from an arable field on the Weald Clay, sample 6 from an old arable soil on the Upper Greensand, and samples 9–11 from small experimental plots receiving annual additions of nitrogen, phosphorus and potassium on soil derived from Keuper Sandstone. Samples 7 and 8 were from mineral soil underlying organic surface mats on old permanent pasture, sample 8 being from the unlimed half of plot 11 of the Park Grass experiment at Rothamsted. Sample 12 was from soil used for several years in a tomato greenhouse, sample 13 from a soil under temporary ley and sample 14 from an arable soil derived from London Clay. All samples were taken to depths of 6 or 8 in.

Table 1. *Analysis of soils*

No.	Soil	pH	Percentage on moisture-free basis		
			C	N	$CaCO_3$
1	Clay loam	8·2	0·99	0·11	2·1
2	Clay loam	7·8	2·51	0·26	1·4
3	Clay loam	7·7	1·09	0·12	0·6
4	Clay loam	7·9	1·13	0·12	0·6
5	Silty clay loam	7·5	2·63	0·26	0·2
6	Medium sandy loam	5·8	0·80	0·07	0
7	Coarse sandy loam	4·1	2·96	0·23	0
8	Clay loam	3·6	3·14	0·33	0
9	Fine sandy loam	4·4	1·81	0·18	0
10	Fine sandy loam	6·5	1·67	0·16	0·1
11	Fine sandy loam	7·9	1·71	0·15	2·4
12	Greenhouse	7·0	5·71	0·54	1·8
13	Medium sandy loam	6·9	3·58	0·38	0·3
14	Heavy clay loam	6·2	3·69	0·34	0·1

Before use the samples were air-dried and ground to pass an 0·5 mm. sieve. Carbon was determined by a modification of the wet combustion technique of Clark & Ogg (1942), nitrogen by the modified Olsen method described below, pH by the glass electrode (soil:water ratio, 1:2) and $CaCO_3$ by the method of Schollenberger (1930).

METHODS

Determination of total-N

If leaching is prevented, loss of nitrogen due to denitrification on addition of nitrate (or nitrite) to soil can be followed by nitrogen determinations provided the method of analysis used is capable of recovering all the non-gaseous compounds of nitrogen likely to be present during the process of denitrification. Investigations on the denitrification of nitrate by bacteria in culture media have shown that the only non-gaseous forms of nitrogen detectable in significant amounts during this process are nitrate-, nitrite-, ammonium- and organic-N, and work described below has established that this holds for the denitrification of nitrate in soil. Small amounts of hydroxylamine have occasionally been found in cultures of denitrifying bacteria, but the other nitrogen compounds postulated as products of dissimilatory nitrate reduction (e.g. hyponitrous acid and imido-nitric acid) are very unstable and they have never been detected in denitrifying systems.

Ammonium- and organic-N can be determined satisfactorily by the normal Kjeldahl procedure, but nitrate and nitrite are not recovered by this method. However, the normal Kjeldahl procedure does include some of the nitrate-N present in a soil sample. This is presumably due to the presence of organic matter in soil since the loss of nitrogen during Kjeldahl digestion of potassium nitrate can be reduced by the addition of organic substances such as glucose. For example, we found that when samples of potassium nitrate containing 5, 10 and 15 mg. of NO_3-N were treated with 0·5 g. of glucose and digested with H_2SO_4 and catalyst as in the normal Kjeldahl method, the recoveries of NO_3-N as NH_4-N were 82, 68 and 56 % respectively. A search of the literature for methods of determining nitrogen which included nitrate and nitrite revealed that Olsen (1929) had described a method for the determination of total nitrogen in soil involving oxidation of nitrite to nitrate by acidified permanganate followed by reduction of nitrate to ammonia with reduced iron and sulphuric acid and subsequent digestion with concentrated sulphuric acid and catalyst as in the normal Kjeldahl procedure. This method seemed well suited for studies on denitrification and its scope and accuracy under conditions likely to be encountered in the denitrification work planned were therefore examined. It was found that with

slight modifications the method gave very satisfactory results and the following modified technique was adopted for total-N determinations.

Modified Olsen method. The soil sample with approximately 10 ml. of H_2O contained in a Kjeldahl flask (300–500 ml.) is treated with 10 ml. of 5 % (w/v) $KMnO_4$, and 20 ml. of 50 % (v/v) H_2SO_4 is added slowly with shaking. After 5 min., two drops of octyl alcohol and 5 g. of reduced iron passing a 100-mesh sieve are added and a 25 ml. Erlenmeyer flask is inverted in the neck of the flask. The flask is shaken and after the initial strong effervescence has subsided (about 15 min.) it is transferred to a Kjeldahl digestion rack with the Erlenmeyer flask still in the neck and heated gently for 45 min., care being taken to ensure that only slight loss of water by evaporation occurs during this process. The flask is allowed to cool, the Erlenmeyer flask is removed, and 10 g. of K_2SO_4, 1 g. of $CuSO_4.5H_2O$, 0·1 g. of Se and 40 ml. of concentrated H_2SO_4 are added. The mixture is heated until it assumes a yellowish green colour and is subsequently boiled gently for 5 hr. The ammonia in the digest is determined by distillation of an aliquot with sodium hydroxide.

In most of the work reported below the above technique was applied to 5 g. samples of soil which had received 5 mg. of N in the form of KNO_3, but satisfactory results were obtained in experiments with up to 20 g. of soil (using 60 ml. of concentrated H_2SO_4 for the Kjeldahl digestion) or as much as 100 mg. of nitrate-N. The volume of water present before the addition of permanganate is not critical as satisfactory results were obtained when 8 or 12 ml. of water were present. The reduced iron supplied by British Drug Houses Ltd. and Hopkin and Williams Ltd. was found to contain significant amounts of nitrogen, but this caused no difficulty as consistent results were obtained in control analyses using 5 g. samples of material passing a 100-mesh sieve.

The results given in Table 2 show that when this modified Olsen procedure is applied to soils containing normal low levels of mineral nitrogen it gives highly consistent results which are in close agreement with those obtained by the normal Kjeldahl method in which the pre-treatments with permanganate and reduced iron in dilute sulphuric acid are omitted. These results were obtained with 5 g. samples of soil ground to pass an 0·5 mm. sieve. Consistent results were also obtained with most of the soils using material ground to pass a 2 mm. sieve, but reproducible results could not be obtained with soils containing a high proportion of medium or coarse sand (e.g. soils 6 and 7) unless samples from material ground to pass an 0·5 mm. sieve were employed. For this reason all soils used in the work reported here were ground to pass an 0·5 mm. sieve.

The recoveries by the modified Olsen method of nitrate-, nitrite- and ammonium-N added to 5 g. samples of various soils as KNO_3, $NaNO_2$, $(NH_4)_2SO_4$, or mixtures of these, in 10 ml. of water are given in Table 3.

Table 2. *Nitrogen contents of various soils as determined by normal Kjeldahl and modified Olsen methods*

	N content (%)*			
	Kjeldahl method		Modified Olsen method	
Soil	Mean of six determinations	Range of determinations	Mean of six determinations	Range of determinations
1	0·107	0·106–0·108	0·107	0·106–0·108
2	0·256	0·254–0·257	0·256	0·255–0·258
3	0·123	0·122–0·124	0·124	0·123–0·124
4	0·124	0·123–0·125	0·124	0·123–0·125
5	0·262	0·260–0·264	0·262	0·261–0·264
6	0·070	0·069–0·072	0·070	0·069–0·071
7	0·229	0·227–0·230	0·230	0·228–0·231

* Moisture-free basis.

It can be seen that the recoveries obtained, each of which represents the mean of three highly consistent results, were practically quantitative with every soil examined. These recovery tests left little doubt that the modified Olsen method would give reliable results if used to follow loss of nitrogen from soil by denitrification but as the investigations planned involved the addition of glucose, straw and other organic substances to soil together with nitrate, experiments were carried out to determine the effect of these substances on the recovery of nitrate-, nitrite- and ammonium-N by the modified Olsen method. The results showed that recovery by this method was not affected by such materials even when they were present in amounts considerably greater than those to be used in the work planned. For example, the recoveries of 5 mg. of nitrogen added as KNO_3 or as $NaNO_2$ to 5 g. samples of soils 1, 3 and 5 were not affected by the presence of 1 g.

of glucose, sawdust or lignin or of 2 g. of wheat straw. Analyses also showed that when the total-N contents of straw, grass, sawdust and other organic materials were determined by the modified Olsen method the results were in close agreement with those given by the normal Kjeldahl procedure.

Since the Olsen method has not been used previously to follow total-N changes due to loss of nitrogen from soil by denitrification it is appropriate to draw attention to some of its advantages over the method most favoured for this purpose, namely the salicylic acid modification of the Kjeldahl method. One advantage is that it can be applied to moist or waterlogged soils, whereas various workers (e.g. Piper, 1944) have reported that the salicylic acid method is affected by water and that to include nitrate by this method the soil must be dried before analysis. We studied the effect of water on the salicylic acid method described by Murneek & Heinze (1937) and found that the recoveries by this method of 10 mg. of NO_2-N (as $NaNO_2$) and of NO_3-N (as KNO_3) added in 10 ml. of water to 5 g. of soil 4 were 54 and 75 % respectively. The fact that water interferes with the salicylic acid method also precludes the use of the preliminary soaking of soil with water which Bal (1925) found necessary to obtain satisfactory results when analysing heavy clay soils by the Kjeldahl method. The necessity of drying the soil sample before analysis by the salicylic acid method involves the risk of loss of nitrate, nitrite and ammonia during the drying process. This danger does not appear to have been appreciated by workers on denitrification, although Broadbent (1951) did carry out some tests in which he added ammonia to soil at pH 7·5 and 8·2 prior to drying at 105° C. and found that loss of ammonia on drying did not exceed 7 %. The fact that serious loss of nitrate, nitrite and ammonia can occur when soil is dried at 105° C. is illustrated by the results given in Table 4.

Table 3. *Recoveries by modified Olsen method of nitrate-, nitrite- and ammonium-*N *added to various soils*

Soil	N in soil (mg.)	N added to soil (mg.)			Total-N (mg.)		Recovery of N added to soil (%)
		NO_3-N	NO_2-N	NH_4-N	Calculated	Found	
1	5·23	5	0	0	10·23	10·20	99·4
1	5·23	0	5	0	10·23	10·19	99·2
1	5·23	5	5	0	15·23	15·20	99·7
1	5·23	5	5	5	20·23	20·18	99·7
2	12·50	5	0	5	22·50	22·42	99·2
2	12·50	5	5	5	27·50	27·50	100·0
3	6·02	5	5	0	16·02	16·00	99·8
3	6·02	5	5	5	21·02	21·10	100·5
4	6·05	5	5	0	16·05	15·98	99·3
4	6·05	5	5	5	21·05	21·02	99·8
5	13·00	5	5	0	23·00	22·90	99·0
5	13·00	5	5	5	28·00	27·93	99·5
6	3·47	5	0	0	8·47	8·40	99·6
6	3·47	5	5	5	18·47	18·39	99·5
7	11·40	5	0	0	16·40	16·40	100·0
7	11·40	0	5	0	16·40	16·34	98·8
7	11·40	5	5	5	26·40	26·42	100·1

Table 4. *Effect of drying on recoveries by modified Olsen method of nitrite-, nitrate- and ammonium-*N *added to soil*

(5 g. samples of soil in Kjeldahl flasks were treated with 10 ml. of a solution containing 5 mg. of nitrogen as sodium nitrite, potassium nitrate or ammonium sulphate or with 10 ml. of a solution containing 5 mg. of nitrogen in each of these three forms. Total-N was determined by the modified Olsen method before and after drying at 105° C. for 16 hr.)

Form of N added	Recovery of N (%)			
	Soil 1 (pH 8·2)		Soil 7 (pH 4·1)	
	Before drying	After drying	Before drying	After drying
Nitrite	99·6	39·6	98·2	11·2
Nitrate	100·0	96·8	99·0	76·8
Ammonium	99·8	41·8	99·3	87·8
Nitrite, nitrate and ammonium	100·0	45·0	98·8	57·0

A further point of relevance here is that the literature does not appear to contain any evidence that the salicylic acid modification of the Kjeldahl method includes nitrite as well as nitrate. Such evidence is clearly required if the method is to be used with confidence to follow total-N changes in a system containing nitrate undergoing reduction to nitrite.

In concluding this section on the determination of total-N in soil it may be pointed out that although the Kjeldahl method is invariably employed for the determination of organic nitrogen in soil, Dyck & McKibbin (1935) have reported that not all the nitrogen in organic soils is determinable by the Kjeldahl method. They analysed twenty-six organic soils by the Dumas and Kjeldahl methods and found that with every sample tested the Dumas method gave a considerably higher percentage of nitrogen. Since this work was confined to organic soils we considered it important to discover if similar results were obtained with mineral soils and in conjunction with Dr T. Breyhan (Institut für Biochemie des Bodens, Forschungsanstalt für Landwirtschaft, Völkenrode, Braunschweig, Germany) we analysed various mineral and organic soils by the macro-Kjeldahl and the micro-Dumas techniques. It was found that with mineral soils the two methods gave practically identical results but that with organic soils the Dumas technique gave values 10–20% higher than those obtained by the Kjeldahl method. The latter observation, which confirmed the findings of Dyck and McKibbin, suggested that the Kjeldahl method was unreliable when applied to organic soils, but further work showed that when occluded air was removed from such soils before analysis by placing them alternately in a vacuum and in an atmosphere of carbon dioxide and repeating the process several times, the values then obtained by the Dumas technique were considerably reduced. Further work using a macro-Dumas instead of a micro-Dumas apparatus in order to reduce errors due to soil sampling, together with a modification of this apparatus which will permit the removal of occluded air from the sample before analysis, is required to complete this investigation, but the indications are that the higher values obtained by the Dumas method with organic soils are due to occlusion of atmospheric nitrogen by the organic matter in these soils.

*Determination of nitrate-, nitrite- and ammonium-*N

The determination of nitrate- and ammonium-N in soil containing nitrate undergoing denitrification is complicated by the fact that nitrite is likely to be present in substantial quantity (see Fig. 5). This precludes the use of the acid extractants generally employed when the simultaneous determination of ammonia and nitrate is required, since they lead to the destruction of nitrite with partial conversion to nitrate (see Bremner & Shaw, 1955). This fact appears to have been overlooked by several workers. For example, Buckett, Duffield & Milton (1955) have recently proposed a method for the determination of nitrite and nitrate in soil in which the nitrate and nitrite are extracted with 10% (v/v) acetic acid at 70° C. We have tested this method and have found that the acetic acid treatment leads to extensive destruction of nitrite with concomitant formation of nitrate in the presence or in the absence of soil. For example, it was found that only 5–30% of nitrite-N added to soil as $NaNO_2$ in amounts ranging from 10 to 1000 p.p.m. could be recovered by this method, the recovery decreasing with increase in the amount of nitrite-N added. The recoveries in the absence of soil varied from 10 to 40%. Analysis of the acetic acid extracts after treatment with sulphamic acid to destroy nitrite showed that as much as 50% of nitrite-N added to soil before extraction with acetic acid was converted to nitrate-N during the extraction. Conway (1947) states that interference due to nitrite in the determination of nitrate by his micro-diffusion technique using Devarda's alloy can be eliminated by acidifying the solution to about pH 1 and exposing it to the atmosphere for 1 hr., but he does not report any results obtained by this procedure. Using this technique with a solution containing 100 μg. of NO_2-N we found that while it destroyed 97–98% of the nitrite it also led to the conversion of 10–12% of the nitrite to nitrate.

The above difficulties were discussed in an earlier paper (Bremner & Shaw, 1955), and it was shown there that when N-K_2SO_4:H_2SO_4 solution is used for the extraction of ammonia and nitrate from soil the conversion of any nitrite present to nitrate can be avoided by the addition of sulphamic acid to the extractant. This technique has proved very satisfactory but it has the disadvantage that a separate extraction with a neutral reagent is necessary if the nitrite-N content of the soil is required. This disadvantage precludes its use in work with waterlogged soils in which subsamples cannot be taken and as such work is essential in studies on denitrification it was necessary to find another reagent which would extract nitrate, nitrite and ammonia quantitatively from soil without decomposing nitrite in the process and which would not interfere with the determination of these three forms of nitrogen. Tests with soils 1, 2, 4 and 11 showed that both N-KCl and N-K_2SO_4 met these requirements. For example, when nitrogen was added to these soils in the form of ammonium sulphate, sodium nitrite and potassium nitrate at the rate of 500 p.p.m. of each form of nitrogen and the soils were then extracted with N-KCl or N-K_2SO_4, the recoveries of the nitrite-N added, as determined by the colorimetric method of Shinn (1941), ranged from 98 to 100%, and the

recoveries of the ammonia- and nitrate-N added, as determined by microdiffusion methods previously described (Bremner & Shaw, 1955) were between 98 and 101 %. Interference due to nitrite in the determination of nitrate in these recovery tests was eliminated by the addition of sulphamic acid to the aliquots taken for nitrate analysis and this technique was adopted for all work involving the determination of nitrate-, nitrite- and ammonium-N, the standard procedure being to shake the soil with N-KCl or N-K_2SO_4 for 2 hr., filter, remove an aliquot of the filtrate for determination of nitrite by the method of Shinn and add sulphamic acid to another aliquot of the filtrate and use this nitrite-free solution for the determination of ammonia and nitrate by microdiffusion methods (Bremner & Shaw, 1955). The volume of N-KCl or N-K_2SO_4 used for extraction was decided from the amounts of ammonia, nitrate and nitrite likely to be present in the sample, but it was never less than 10 ml./g. of soil. Owing to the sensitivity of the colorimetric method of nitrite determination it was necessary to make dilutions of the aliquots for determination of nitrite-N. Nitrite was removed from the aliquots taken for ammonia and nitrate determinations by treating 10 ml. with 1 ml. of 2 % (w/v) sulphamic acid solution and shaking vigorously for 2–3 min.

The advantages of the microdiffusion methods have already been discussed (Bremner & Shaw, 1955), but it may be pointed out here that the microdiffusion method of determining nitrate is particularly well suited for denitrification studies involving the addition of both nitrate and organic materials to soil since it does not appear to be affected by organic matter. For example, tests showed that the recovery by the microdiffusion method of 100 μg. of NO_3-N was not affected by the presence of as much as 25 mg. of glucose. The colorimetric phenoldisulphonic acid method of determining nitrate is seriously affected if the solution analysed contains an appreciable amount of organic matter and various workers (e.g. Panganiban, 1925; Karlsen, 1938) have found that for this reason it could not be employed in work on denitrification.

RESULTS

Recovery of nitrate after incubation with moist and waterlogged soils

The recoveries of nitrate added to the soils chosen for this investigation after incubation at two moisture levels for 80 days at 25° C. are given in Table 5. The soil samples (20 g.) were treated with 100 p.p.m. of nitrate-N (as KNO_3) and incubated in stoppered bottles at moisture levels corresponding to 60 and 120 % of their water-holding capacity. Water-holding capacity was calculated from the amount of water retained when 20 g. of air-dried soil placed in a filter funnel plugged with a wisp of cotton wool were treated with 20 ml. of water and allowed to drain overnight with the funnel covered with a clock glass. The bottles were aerated at 4-day intervals and after 80 days the entire contents of each bottle were extracted with N-K_2SO_4 and the nitrate-, nitrite- and ammonium-N contents of the extracts determined by the methods described above. The recovery of added nitrate after 80 days was calculated by subtracting the nitrate-N found in control experiments in which no nitrate was added to the soil from the nitrate-N found in the soil which had received nitrate. Considerable amounts of ammonia were found in some of the soils after incubation with nitrate, but since identical amounts were found in the control experiments in which no nitrate was added it seems safe to assume that this ammonia was not derived by reduction of the added nitrate. Nitrite was not detectable in any of the soils after incubation for 80 days.

Table 5. *Recoveries of nitrate added to soils after incubation at two moisture levels*

(Soils were incubated with 100 p.p.m. of nitrate-N (as KNO_3) at two moisture levels for 80 days at 25° C.)

	Moisture level (as % of W.H.C.*)	
	60	120
Soil	Recovery of nitrate (%)	
1	100	94
2	95	20
3	91	88
4	100	99
5	53	0
6	99	98
7	98	40
8	98	0
9	100	55
10	94	8
11	94	23
12	88	0
13	46	0
14	88	2

* Water-holding capacity of air-dried soil.

It can be seen that extensive loss of nitrate occurred in only two of the soils (nos. 5 and 13) when the soils were incubated at a moisture level corresponding to 60 % of their water-holding capacity, but that it occurred in ten of the fourteen soils examined when the soils were waterlogged.

Loss of nitrogen from waterlogged soil incubated with nitrate

Loss of nitrate in the experiments described above could not be taken as evidence of denitrification since it could have been due to microbial assimilation of nitrate, a process which does not involve gaseous

loss of nitrogen. Experiments were therefore carried out to discover if loss of nitrogen due to denitrification could be detected by total-N analysis after incubation with nitrate. The technique used in these experiments was as follows. Eleven ml. of water containing 5 mg. of NO_3-N (as KNO_3) were added to 5 g. samples of soil contained in 300 ml. Kjeldahl flasks and the flasks were stoppered and incubated at 25° C. After 20 days the entire contents of each flask were analysed for total-N by the modified Olsen method described above, loss of nitrogen being calculated from similar total-N determinations on the soil sample plus nitrate at the beginning of the experiment. Since the four soils which lost little nitrate under waterlogged conditions were those with the lowest carbon contents (cf. Tables 1 and 5) another series of experiments in which glucose (125 mg.) was added to the soil-nitrate mixtures was performed to discover the effect of adding a readily decomposable organic compound. The results (Table 6) showed that in the absence of glucose no loss of nitrogen due to denitrification could be detected with the four soils of low carbon content (nos. 1, 3, 4 and 6) but that loss of nitrogen was readily detectable with the three soils of higher carbon content tested (nos. 5, 12 and 13). They also showed that extensive loss of nitrogen was induced by the addition of glucose to the soils of low carbon content and that loss of nitrogen from the other soils was increased by this addition.

It may be noted here that, unless otherwise stated, the technique used in the experiments described above was adopted in all subsequent investigations on the denitrification of nitrate in waterlogged soil and that all results presented were derived from duplicate or triplicate experiments.

Loss of nitrogen from waterlogged soil incubated with nitrate and different amounts of glucose

The results given in Table 6 showed that to study the factors affecting denitrification of nitrate in soil by total-N determinations it would be necessary to add some organic material with the nitrate. Experiments were therefore carried out to determine the effect of adding different amounts of glucose to waterlogged soil containing a fixed level of added nitrate. The results (Table 7 and Fig. 1*a*) showed that with the exception of soil 6, which had a lower carbon content than the other soils tested, maximal loss of nitrogen occurred when the ratio of carbon added as glucose to nitrogen added as nitrate was 2:1 or 3:1. Further experiments using different amounts of finely ground wheat straw showed that, as with glucose, loss of nitrogen increased and then decreased with increase in the amount added (Fig. 1*b*). The only apparent explanation of these results was that glucose or straw added to soil in excess of the amount required by the denitrifying organisms for denitrification of the nitrate present is used by other organisms for the fixation of atmospheric nitrogen. Evidence for this was obtained by following the rate of loss of nitrogen from soil 4 when it was waterlogged and treated with nitrate and different amounts of wheat straw. The results (Fig. 2) showed that whereas the loss of nitrogen continued to increase with time of incubation when small amounts of straw were added, there was a distinct decrease in the loss of nitrogen after a certain period of incubation with large amounts of straw. Similar results were obtained in experiments with glucose and further tests showed that if extra glucose was added to a waterlogged soil previously incubated with nitrate and glucose for 6 days, the loss of nitrogen after further incubation for 24 days was less than the loss at 6 days (Table 8).

Fixation of nitrogen on incubation of waterlogged soils with glucose in the absence of nitrate is demonstrated by the results given in Table 9. It is note-

Table 6. *Loss of nitrogen on incubation of waterlogged soils with nitrate in the presence and absence of glucose*

(5 g. samples of soil were treated with 10 ml. KNO_3 solution (5 mg. NO_3-N) and incubated with or without glucose (50 mg. C) at 25° C. for 20 days.)

	N loss (as % of added NO_3-N)	
Soil	Glucose absent	Glucose present
1	0	80
3	0	76
4	0	69
6	0	67
5	20	77
12	37	73
13	80	90

Table 7. *Loss of nitrogen on incubation of waterlogged soils containing nitrate and different amounts of glucose*

(5 g. samples of soil were incubated at 25° C. for 20 days with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) and different amounts of glucose.)

C/N ratio of glucose-nitrate solution added	N loss (as % of added NO_3-N)					
	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6
1	41	54	32	35	72	7
2	80	98	80	77	101	22
3	90	97	91	90	95	40
4	86	94	89	88	92	68
5	82	88	80	83	83	69
10	80	75	76	69	77	67
20	71	70	70	66	70	60
50	59	63	69	61	68	46

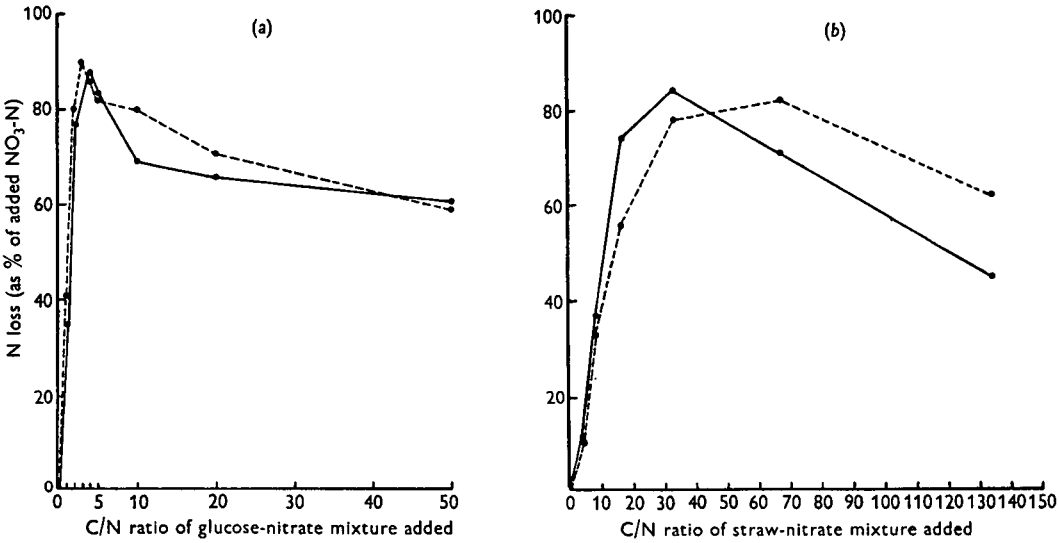


Fig. 1. Loss of nitrogen from waterlogged soils incubated with nitrate and different amounts of glucose or wheat straw. 5 g. samples of soil were treated with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) and incubated with different amounts of glucose (*a*) or wheat straw (*b*) for 20 days at 25° C. Wheat straw contained 43·1 % carbon and 0·47 % nitrogen (moisture-free basis). ●—●, Soil 1; ●----●, Soil 4.

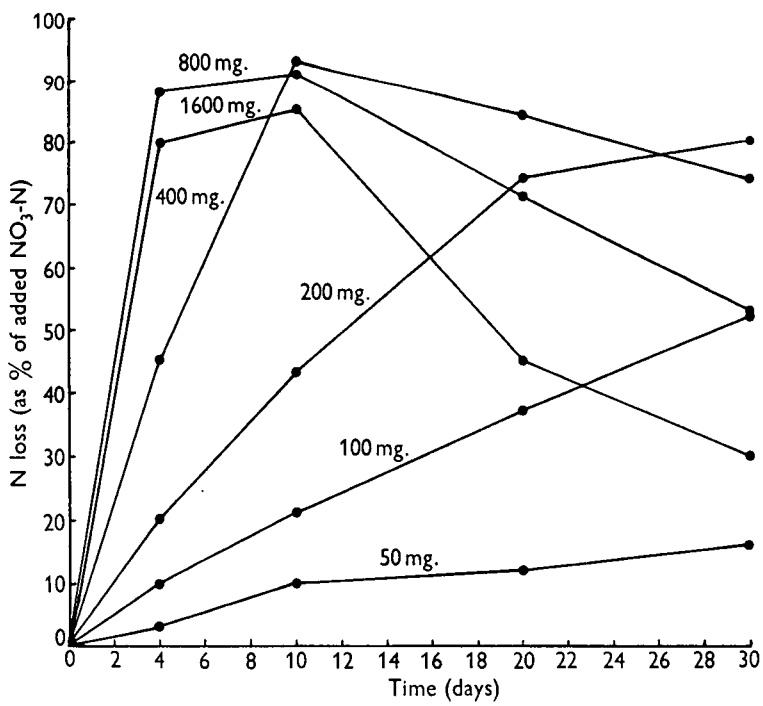


Fig. 2. Loss of nitrogen from soil incubated with nitrate and different amounts of wheat straw. 5 g. samples of soil 4 were mixed with 50, 100, 200, 400, 800 or 1600 mg. wheat straw and incubated with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) at 25° C.

worthy that whereas nitrogen fixation was detectable at all levels of glucose with soils 4 and 6 it was not detectable with soil 5 until high levels of glucose were added. However, comparison of the results obtained with this soil when glucose was absent and when this soil is incubated with nitrate and glucose is derived from the soil itself, presumably by a process involving denitrification of nitrate (or nitrite) formed by nitrification of organic nitrogen compounds during incubation (see Table 7 and Part II, Fig. 1).

Table 8. *Effect of adding different amounts of glucose to waterlogged soil previously incubated with nitrate and glucose*

(Fifteen 300 ml. Kjeldahl flasks each containing 5 g. samples of soil 4 treated with 5 mg. NO_3-N (as KNO_3) and 37·5 mg. of glucose (15 mg. C) dissolved in 11 ml. water were incubated at 25° C. After 6 days the contents of three of the flasks were analysed for total-N and glucose was added to the other twelve flasks at four levels (0, 88, 213 or 463 mg.), three flasks being used for each level. The twelve flasks were then incubated at 25° C. for a further 24 days.)

Glucose added after 6 days' incubation (mg.)*	Period of incubation (days)		
	0	6	30
	Total-N (mg.)		
0 (15)	11·05	6·95	6·35
88 (50)	11·05	6·95	7·05
213 (100)	11·05	6·95	7·30
463 (200)	11·05	6·95	7·60

* Figures in parentheses are total amounts of carbon added to soil as glucose including addition (15 mg. C) before incubation.

Table 9. *Total*-N *changes on incubation of waterlogged soils with glucose*

(5 g. samples of soil were incubated at 25° C. for 20 days with 11 ml. water containing different amounts of glucose.)

	Glucose added (mg.)	Total-N (mg.) Before incubation	Total-N (mg.) After incubation	Gain of N (mg.)
Soil 4	0	6·04	6·00	—
	37·5	6·04	6·44	0·40
	62·5	6·04	6·55	0·51
	125·0	6·04	6·88	0·84
	250·0	6·04	7·02	0·98
	500·0	6·04	7·24	1·20
Soil 5	0	13·00	12·30	—
	25·0	13·00	12·75	—
	37·5	13·00	12·90	—
	62·5	13·00	12·98	—
	125·0	13·00	13·16	0·16
	250·0	13·00	13·31	0·31
	625·0	13·00	13·40	0·40
Soil 6	0	3·48	3·46	—
	62·5	3·48	3·63	0·15
	125·0	3·48	4·04	0·56
	250·0	3·48	4·17	0·69
	500·0	3·48	4·33	0·85

when it was present at low levels suggests that fixation of nitrogen did occur with low levels of glucose. The finding that loss of nitrogen was detectable after incubation of soil 5 with water in the absence of nitrate or glucose supports other indications that a significant fraction of the nitrogen lost

Further experiments showed that the amounts of nitrogen fixed when 5 g. samples of soils 1, 3 and 4 were incubated with 250 mg. of glucose under waterlogged conditions were practically the same as the amounts fixed when the soils were incubated at moisture levels corresponding to 50 % of their water-holding capacity. For example, when soil 4 was incubated with glucose at moisture levels corresponding to 50, 100 and 500 % of its water-holding capacity for 20 days at 25° C. the amounts of nitrogen fixed were 1·02, 1·09 and 1·01 mg., respectively.

Nitrogen fixation was also detected in experiments with straw. For example, it was found that when 5 g. samples of soil 4 were incubated with 12 ml. of water and different amounts of wheat straw for 30 days at 25° C., 0·02, 1·72 and 2·02 mg. of nitrogen were fixed using 250, 800 and 1600 mg. of straw, respectively.

The work reported above showed that if denitrification of nitrate in soil is followed by total-N determinations the results may be seriously affected by nitrogen fixation if the amount of organic material added to induce denitrification is much in excess of the amount required by the denitrifying organisms. In all subsequent work, therefore, the amount of glucose added to induce denitrification of nitrate in the different soils was that found to lead to maximal loss of nitrogen (see Table 7).

Loss of nitrogen from soil incubated with different levels of nitrate

The effect of varying the level of nitrate added to waterlogged soil on subsequent loss of nitrogen by denitrification was studied in two series of experiments. In one, different amounts of nitrate were added to a fixed weight of soil: in the other, a fixed amount of nitrate was added to different weights of soil. Since the work reported in the previous section showed that loss of nitrogen varied with the level of glucose added with the nitrate, glucose was added in both series of experiments in amounts such that the ratio of carbon added as glucose to nitrogen added as nitrate was constant at 3:1, this ratio being chosen because it gave maximal loss of nitrogen with the soils (nos. 1 and 4) used in this investigation (see Table 7). The results obtained when different amounts of nitrate were added to a fixed weight of soil (Table 10) showed that the loss of nitrogen after 10 days, calculated as a percentage of the NO_3-N added, did not vary with the level of nitrate. They also showed that the rate of loss of

Table 10. *Loss of nitrogen from waterlogged soil incubated with different levels of nitrate*

(5 g. samples of soil 4 were incubated at 25° C. with 10 ml. KNO_3-glucose solution (C/N ratio, 3) containing 2·5, 5·0, 7·5, 10·0 or 20·0 mg. NO_3-N.)

NO_3-N added (mg.)*	Glucose-C added (mg.)	Period of incubation (days): 4 — N loss (as % of added NO_3-N)	Period of incubation (days): 10 — N loss (as % of added NO_3-N)
2·5 (500)	7·5	79	81
5·0 (1000)	15·0	80	83
7·5 (1500)	22·5	81	83
10·0 (2000)	30·0	79	82
20·0 (4000)	60·0	40	84

* Figures in parentheses are amounts of N added expressed in p.p.m. of air-dried soil.

Taken together, the results of these two series of experiments showed that when nitrate was added to soil at any level from 250 to 2000 p.p.m., the rate of loss of nitrogen (calculated as a percentage of the NO_3-N added) was not affected by the level of nitrate. The rate of loss of nitrogen with lower levels of nitrate could not be determined accurately by total-N analysis, but the results given in Table 10 and Fig. 3 suggest that it should not differ appreciably from the rate with levels of 250–2000 p.p.m. of nitrate-N.

The finding that the rate of loss of nitrogen after addition of 5 mg. of NO_3-N to different weights of soil was very slow with 0·1 g. of soil, and that it increased with weight of soil up to 5 g. and thereafter remained constant was explicable on the grounds that if less than 5 g. of soil were used the quantity of readily available nutrients (iron, copper, manganese, phosphorus, etc.) supplied by the soil for the growth of denitrifying organisms was too limited to permit rapid denitrification of 5 mg. of NO_3-N. Other possible explanations were that when less than 5 g. of soil were used the amount of soil added did not contain sufficient numbers of denitrifying bacteria for rapid denitrification of 5 mg. of

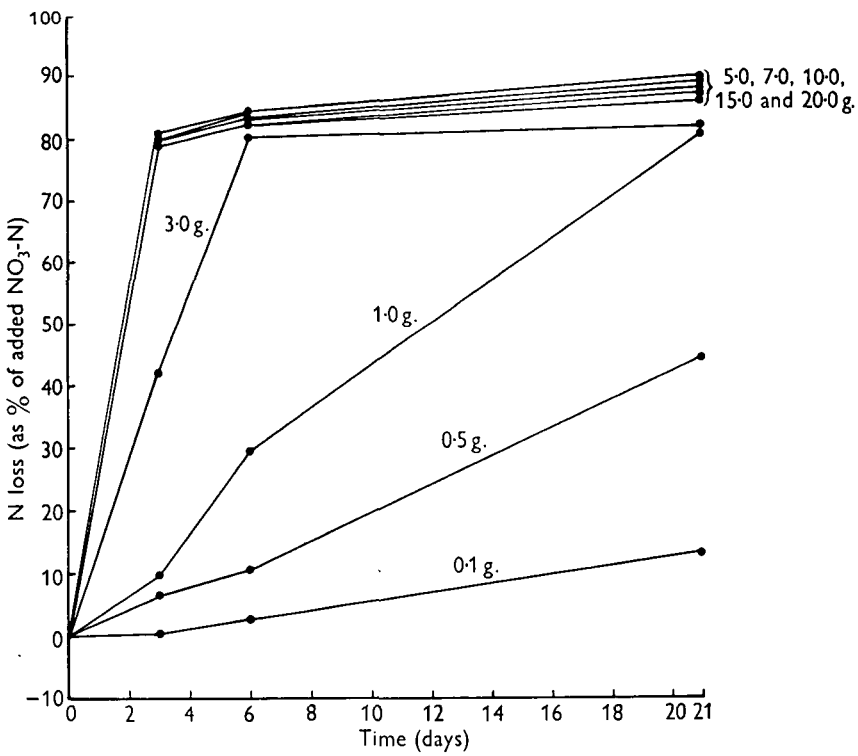


Fig. 3. Rate of loss of nitrogen on incubation of different amounts of soil with the same amounts of nitrate and glucose. Flasks containing 5 mg. NO_3-N (as KNO_3) and 15 mg. C (as glucose) dissolved in 11 ml. water were treated with different amounts of soil 1 and incubated at 25° C. Amount (g.) of soil added is given on each graph.

nitrogen was affected by the level of nitrate-N only when the latter was very high (4000 p.p.m.). The results obtained when a fixed amount of nitrate was added to different weights of soil (Fig. 3) showed that the rate of loss of nitrogen after the addition of 5 mg. of NO_3-N to soil increased with weight of soil up to 5 g. and thereafter remained constant.

nitrate-N or did not possess sufficient buffering capacity to prevent a drop in pH due to the formation of organic acids by microbial decomposition of the glucose present. The latter possibility was eliminated by experiments which showed that no fall in pH occurred when 0·1 g. samples of soil 1 were incubated with 11 ml. of water containing 5 mg. of NO_3-N and 15 mg. of carbon as glucose at 25° C. for 21 days, and that the rate of loss of nitrogen during such incubations with 0·1 g. of soil was not significantly affected by the addition of 1 g. of $CaCO_3$. The question was finally resolved by experiments in which the rate of loss of nitrogen from 5 g. samples of soil treated with 10 ml. of a sterile solution of potassium nitrate and glucose was compared with that from 5 g. samples of sterile soil treated with 10 ml. of the same nitrate-glucose solution and 0·1 g. of unsterilized soil. The results (Fig. 4) showed that 0·1 g. of soil did contain sufficient numbers of denitrifying bacteria for rapid denitrification of 5 mg. of NO_3-N but did not contain an adequate supply of the nutrients required by the denitrifying bacteria for this process. When the necessary nutrients were supplied to 0·1 g. of soil in the form of 5 g. of sterile soil the rate of denitrification was then practically the same as with 5 g. of soil.

Attention may be drawn to results in Fig. 4 which show that although extensive loss of nitrogen occurred when sterile glucose-nitrate solution was added to 5 g. of soil, no loss of nitrogen occurred when the same solution was added to 5 g. of sterile soil. The results of these sterilization experiments, together with the results of other experiments which showed that toluene completely inhibited loss of nitrogen from waterlogged soils containing nitrate and glucose, provide good evidence that the process of denitrification in soil is due to the activities of soil micro-organisms.

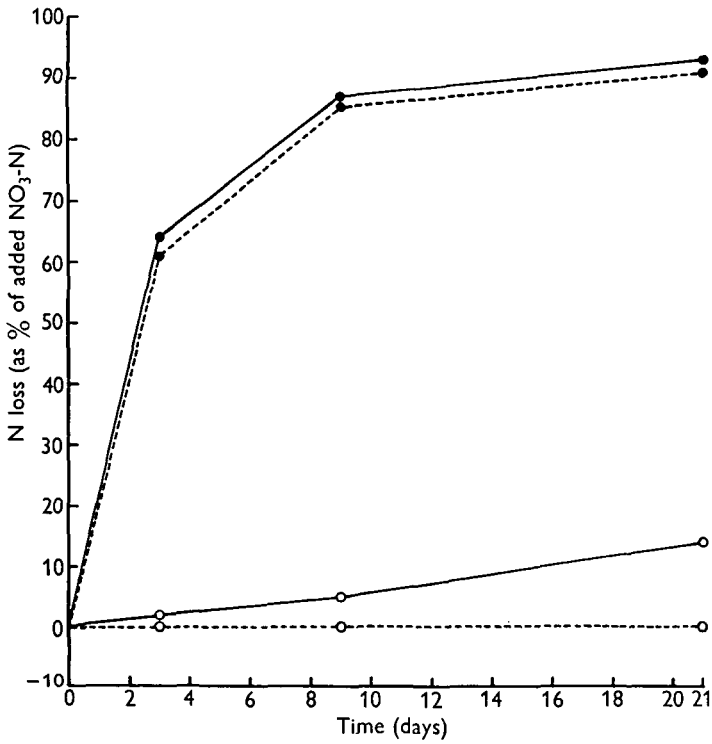


Fig. 4. Loss of nitrogen on incubation of unsterilized soil with nitrate and glucose in the presence and absence of sterile soil. A solution of potassium nitrate and glucose containing 5 mg. NO_3-N and 15 mg. C/10 ml. was autoclaved at 120° C. for 20 min. and 10 ml. aliquots of the sterile solution were transferred aseptically to 300 ml. Kjeldahl flasks containing 5 g. unsterilized soil, 5 g. sterile soil plus 0·1 g. unsterilized soil, 0·1 g. unsterilized soil, or 5 g. sterile soil. Flasks were plugged with cotton wool and incubated at 25° C. Soil used (no. 1) was sterilized by autoclaving at 126° C. for 30 min. ●—●, 5·0 g. unsterilized soil + 10 ml. sterile glucose-nitrate solution; ●- - -●, 5·0 g. sterile soil + 0·1 g. unsterilized soil + 10 ml. sterile glucose-nitrate solution; ○——○, 0·1 g. unsterilized soil + 10 ml. sterile glucose-nitrate solution; ○- - -○, 5·0 g. sterile soil + 10 ml. sterile glucose-nitrate solution.

Nitrogen transformations during the denitrification of nitrate in waterlogged soil

Nitrogen transformations during the denitrification of nitrate in waterlogged soil were followed by determining the amounts of total-, nitrate-, nitrite-, hydroxylamine- and ammonium-N present in soil incubated with nitrate and glucose at 25° C. for various periods of time. The incubations were performed at 25° C. in stoppered 300 ml. Kjeldahl flasks with 5 g. samples of soil treated with 11 ml. of water containing 5 mg. of NO_3-N (as KNO_3) and 15 mg. of carbon (as glucose). After various periods of time six flasks were removed from the incubator, three being used for determinations of total-N and three for determinations of nitrate-, nitrite-, hydroxylamine- and ammonium-N. The latter determinations were made on extracts obtained by shaking the soil

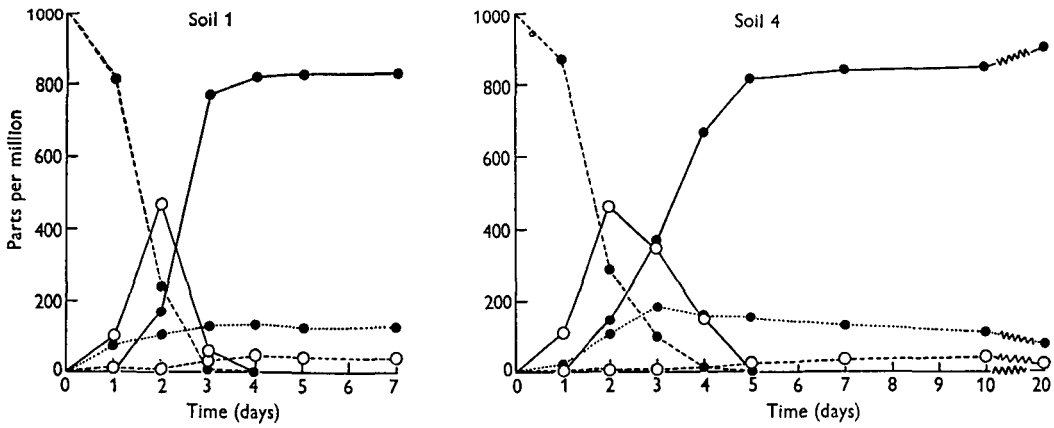


Fig. 5. Nitrogen transformations during denitrification of nitrate in waterlogged soil. 5 g. samples of soil were incubated at 25° C. with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) and 15 mg. C (as glucose). ●—●, N loss; ●- - -●, NO_3-N; ○—○, NO_2-N; ○- - -○, NH_4-N; ●·····●, unidentified N [N added minus N loss minus (NH_4+NO_3+NO_2)-N].

474 p.p.m.) of nitrite-N, but that after a few days no trace of either nitrate or nitrite could be detected. The disappearance of nitrate was also accompanied by the formation of small amounts of ammonia, but it is difficult to decide if this ammonia was derived by reduction of the nitrate added or was produced by ammonification of organic nitrogen compounds either originally present in the soil or synthesized by micro-organisms during the process of denitrification. For this reason the data regarding unidentified-N should be treated with reserve since they were obtained by calculations in which it was assumed that the ammonium-N found after incubation was derived by reduction of the nitrate-N added. The calculations of unidentified-N, which is presumably in the form of microbially-synthesized organic nitrogen compounds, are also based on the assumption that all the nitrogen lost during incubation was derived from the nitrate-N added, but experiments using potassium nitrate labelled with ^{15}N showed that this assumption was valid for the soils tested. For example, experiments in which 5 g. samples of soil 4 were incubated with 11 ml. of water containing 15 mg. of carbon as glucose and 5 mg. of NO_3-N as KNO_3 enriched 52·3 atom % with respect to ^{15}N showed that after 4 or 21 days' incubation at 25° C. the loss of nitrogen was identical to the loss of added NO_3-N as calculated from determinations of the ratio of ^{15}N to ^{14}N in the soil before and after incubation for 4 and 21 days. To determine this ratio, aliquots of the H_2SO_4 digests obtained when the soils were analysed by the modified Olsen method were distilled with alkali, the NH_3 liberated was collected in 0·2 N-HCl, and the N_2 obtained by treatment of the NH_4Cl solution with sodium hypobromite (see Glascock, 1954) was analysed in the mass spectrometer.

samples with N-KCl for 2 hr., ammonia, nitrate and nitrite being determined by the methods previously described and hydroxylamine by the colorimetric method of Csaky (1948), which is based on oxidation of hydroxylamine to nitrite by iodine in acetic acid and estimation of the nitrite with sulphanilic acid and 1-naphthylamine. The mean results of the triplicate analyses performed after different periods of incubation are presented graphically in Fig. 5. Hydroxylamine-N is not included in these graphs since no trace of hydroxylamine could be detected at any stage of incubation. The expression 'unidentified N' is used to describe nitrogen originally added as nitrate which was not lost during incubation and could not be accounted for as nitrate, nitrite, hydroxylamine or ammonia.

It can be seen that with both soils tested the disappearance of nitrate-N was accompanied by the formation of considerable amounts (up to

It is noteworthy that although loss of nitrogen closely followed the disappearance of nitrate and nitrite, it was still detectable after the complete disappearance of both nitrate and nitrite. This finding led us to investigate the possibility that some of the gaseous loss of nitrogen observed on incubation of waterlogged soil containing nitrate and glucose is due to volatilization of ammonia formed either by reduction of the nitrate or by ammonification of organic nitrogen compounds. To test this possibility 5 g. samples of soils 1–7 in 300 ml. Kjeldahl flasks were treated with 11 ml. of water containing 5 mg. of NO_3-N (as KNO_3) and 15 mg. of carbon (as glucose) and a small test-tube ($3 \times \frac{5}{8}$ in.) containing 4 ml. of 50 % (v/v) H_2SO_4 was suspended just above the surface of the liquid in each flask by means of wire attached to the stopper of the flask. The flasks were then incubated at 25° C. and after 20 days the tubes were removed and their contents analysed for NH_4-N by Nesslerization. It was found that although ammonium-N was present in most of the tubes analysed, it never amounted to more than 1 % of the nitrogen added as nitrate. The effectiveness of this method of determining ammonia-N liberated during incubation can be judged from the fact that when flasks containing 10 ml. of ammonium sulphate solution and 2 ml. of 5N-NaOH were incubated at 25° C. for 20 days more than 97 % of the ammonium-N (0·5 mg.) was absorbed by the acid in the tubes. In view of these results the only apparent explanation of the continued loss of nitrogen from waterlogged soil in which neither nitrate nor nitrite is detectable is that nitrate (or nitrite) is produced by nitrification of organic nitrogen compounds and is immediately denitrified. Some support for this explanation is provided by the observation that the continued loss of nitrogen after disappearance of nitrate and nitrite is accompanied by a decrease in the amount of unidentified-N (Fig. 5).

Failure to detect hydroxylamine during the denitrification of nitrate in soil cannot be taken as evidence that this substance is not formed since later work showed that when hydroxylamine-N as hydroxylamine hydrochloride was added at the rate of 200 p.p.m. to the soils used in this work and the soils were immediately extracted with water or N-KCl no trace of hydroxylamine could be detected in the extracts. Other experiments in which 5 mg. of hydroxylamine-N (as $NH_2OH.HCl$) were added to 5 g. samples of soils 1 and 4 containing 10 ml. of water showed that only 70–80 % of the added N could be recovered when the mixtures were immediately analysed for nitrogen by a method which gave quantitative recovery of hydroxylamine-N in the absence of soil, namely the modified Olsen technique with the pretreatment with permanganate omitted. It would appear, therefore, that hydroxylamine is rapidly decomposed in these soils by some non-biological reaction leading to the formation of gaseous products of nitrogen. The mechanism of this reaction was not investigated but it seems probable that it involves higher oxides of manganese and iron since it is known that these react with hydroxylamine with formation of gaseous products of nitrogen.

Experiments in which waterlogged soils containing nitrate and different levels of glucose were incubated at 25° C. for various periods of time showed that nitrite (and nitrate) persisted at low levels of glucose and that ammonia production increased with the level of glucose added. For example, it was found that when 5 g. samples of soil 1 were incubated at 25° C. with 11 ml. of water containing 5 mg. of NO_3-N (as KNO_3) and 5, 15, 25 or 50 mg. of carbon as glucose, the amounts of nitrate-, nitrite- and ammonium-N present after 7 days were as follows:

C added as glucose (mg.)	N found after 7 days (mg.)		
	NO_3-N	NO_2-N	NH_4-N
5	2·05	1·54	0·09
15	0	0	0·20
25	0	0	0·46
50	0	0	0·73

Change in pH *during denitrification of nitrate in soil*

Since numerous workers have shown that the denitrification of nitrate in cultures of bacteria is accompanied by a marked rise in the pH of the culture medium, experiments were carried out to discover if a similar rise in pH could be detected during the denitrification of nitrate in soil. The results showed that an increase in pH did occur when 5 g. samples of soils 1–6 were incubated at 25° C. with 11 ml. of water containing 5 mg. of NO_3-N and the amount of glucose required for maximal denitrification, the most marked increase being obtained with soil 6, which had a lower buffer capacity than the other soils tested. The results obtained with this soil are given in Fig. 6. The pH values after various periods of incubation were determined with the glass electrode, measurements being performed both before and after making the suspensions normal with respect to KCl. The latter was added in order to mask effects due to variation in the amount of KNO_3 present at different stages of incubation.

There seems little doubt that the change in pH observed during the first 4 days of incubation with nitrate and glucose was due to denitrification of the nitrate since analyses performed simultaneously with the pH measurements reported in Fig. 6 showed that no nitrate or nitrite was detectable after 4 days' incubation with nitrate and glucose, and that no denitrification of nitrate occurred in the absence of glucose.

Comparison of loss of nitrogen from systems open and closed to the atmosphere

Since the denitrifying systems investigated above were all closed to the atmosphere it was considered important to discover if the same results were obtained when the systems were open to the atmosphere and free escape of gas and entry of air was permitted. A series of experiments were therefore carried out to compare the loss of nitrogen on incubation of soil with nitrate and glucose under waterlogged conditions in Kjeldahl flasks which were stoppered, unstoppered or stoppered but aerated twice daily by blowing air gently for 3 min. over the surface of the waterlogged soil. The results (Table 11) showed that the rate of loss of nitrogen was the same in the closed as in the open or aerated systems. It is important to note that the contents of the flasks were not shaken or otherwise disturbed during incubation. Experiments reported in Part II of this series show that this leads to a decrease in the rate of loss of nitrogen.

Effect of air-drying and storage of soil

Since the soils employed in the work reported above were always air-dried and usually stored for a considerable period of time before use, experiments were carried out to discover if samples freshly taken from the field gave similar results. For these experiments a large sample of soil (0–6 in.) freshly taken from the field was sieved as rapidly as possible and after thorough mixing, samples of the sieved material were immediately weighed into Kjeldahl flasks, treated with a solution of nitrate and glucose and incubated at 25° C. The remainder of the sieved material was spread out to dry at 25° C. and after 6 days samples of the air-dried material were taken for a second series of incubations similar to those performed with the fresh soil. The residue of the air-dried material was then stored in a stoppered bottle for 6 months and a third series of incubations with nitrate and glucose were performed with this stored material. The results (Table 12) showed that loss of nitrogen on incubation with nitrate and glucose was initially less rapid with fresh than with air-dried or air-dried and stored soil, but that after 5 days' incubation the losses of nitrogen from the three soils were practically identical.

It is of interest in this connexion to record that in studies on the biological activity of soil samples taken from plots 3 and 7 of the Broadbalk continuous wheat field in 1881, and subsequently stored in an air-dried condition in sealed bottles, it was found that the rate of denitrification when these stored soils were waterlogged and treated with nitrate and glucose was even more rapid during the

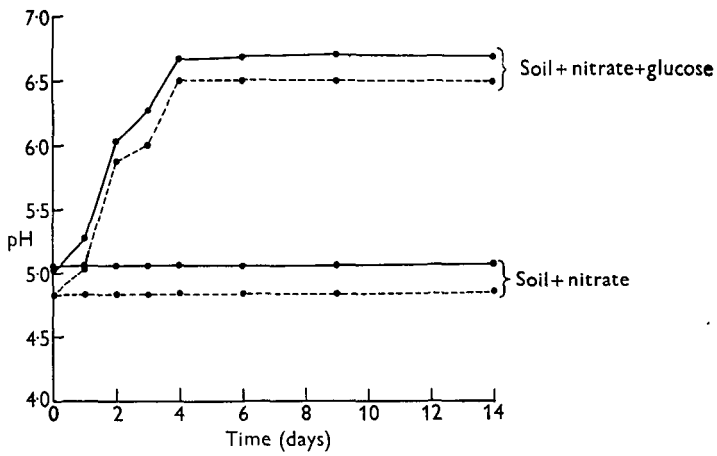


Fig. 6. Change in pH on incubation of waterlogged soil with nitrate in the presence and absence of glucose. 5 g. samples of soil were treated with 5 mg. NO_3-N (as KNO_3) dissolved in 11 ml. water and incubated with or without glucose (25 mg. carbon) at 25° C.

Table 11. *Loss of nitrogen from waterlogged soil incubated with nitrate and glucose in stoppered and unstoppered flasks*

(5 g. samples of soil in 300 ml. Kjeldahl flasks were incubated at 25° C. with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) and 15 mg. C (as glucose). Flasks were stoppered (A), unstoppered (B), or stoppered and aerated twice daily (C).)

		Period of incubation (days)						
		1	2	3	5	10	20	30
		N loss (as % of added NO_3-N)						
Soil 1	A	2	26	76	85	87	90	92
	B	2	25	74	84	86	89	93
	C	1	24	75	85	87	89	91
Soil 3	A	2	35	75	83	86	91	93
	B	1	34	73	83	85	90	92
	C	2	36	75	84	87	92	93

first 5 days of incubation than the rate observed under comparable conditions with soil samples taken from the same plots in 1956 after storage in the air-dried condition for 6 months (Bremner, 1957*a*). It would appear, therefore, that the denitrifying activity of soil is not diminished by storage in the air-dried condition for long periods of time.

Reproducibility of results obtained by incubation technique

The reproducibility of the results obtained in determinations of total-, nitrate-, nitrite and ammonium-N after incubation of soils with nitrate and glucose for various periods of time is illustrated by the results given in Tables 13 and 14. It can be seen that the results obtained in replicate incubations were highly consistent.

Table 12. *Loss of nitrogen on incubation of fresh and air-dried soil with nitrate and glucose under waterlogged conditions*

(5 g. samples of soil were incubated at 25° C. with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) and 15 mg. C (as glucose). Soil used was fresh, air-dried or air-dried and stored for 6 months.)

		Period of incubation (days)						
		1	2	3	5	10	20	30
		N loss (as % of added NO_3-N)						
Soil 1	Fresh	0	11	27	81	84	89	91
	Air-dried	3	26	77	84	87	90	92
	Air-dried and stored	5	30	81	85	88	90	92
Soil 3	Fresh	0	11	30	80	82	88	90
	Air-dried	2	34	73	82	84	90	91
	Air-dried and stored	4	40	77	83	85	90	92
Soil 4	Fresh	6	23	66	79	83	88	91
	Air-dried	12	74	78	80	84	90	93
	Air-dried and stored	14	78	80	82	85	90	92

Table 13. *Reproducibility of results of incubation experiments: loss of nitrogen on incubation of waterlogged soil containing nitrate and glucose*

(5 g. samples of soil 1 in 300 ml. Kjeldahl flasks were incubated at 25° C. with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) and 15 mg. C (as glucose). After various periods of incubation the contents of six flasks were analysed for total-N by the modified Olsen method.)

Period of incubation (days)	N loss (as % of added NO_3-N)*					
3	76·7	77·2	76·0	76·9	77·0	77·3
5	83·6	84·1	83·3	84·3	83·9	84·3
8	84·6	85·0	84·1	85·2	84·2	85·1
12	86·9	87·6	86·4	87·4	86·6	87·5
20	90·3	91·0	90·5	91·1	90·2	91·2

* Results obtained in six replicate experiments.

Table 14. *Reproducibility of results of incubation experiments: nitrate-, nitrite- and ammonium-N in waterlogged soil incubated with nitrate and glucose*

(5 g. samples of soil 4 in 300 ml. Kjeldahl flasks were incubated at 25° C. with 11 ml. water containing 5 mg. NO_3-N (as KNO_3) and 15 mg. C (as glucose); after various periods of incubation the contents of 4 flasks were analysed for nitrate-, nitrite- and ammonium-N.)

Period of incubation (days)	P.p.m.*		
	NO_3-N	NO_2-N	NH_4-N
0	1004	0	3
	1008	0	4
	1010	0	3
	1006	0	5
2	289	461	4
	274	470	5
	293	464	4
	279	465	6
7	0	0	34
	0	0	36
	0	0	30
	0	0	33
20	0	0	21
	0	0	23
	0	0	19
	0	0	21

* Results obtained in four replicate experiments.

DISCUSSION

The results presented in this paper show that to follow loss of nitrogen by denitrification of nitrate in soil by analysis of the soil, it is necessary to employ a method of determining nitrogen which includes both nitrite and nitrate. The modified Olsen method of determining nitrogen described in this paper meets this requirement and it is well suited for investigations on denitrification in soil since it is not affected by the presence of considerable amounts of water or organic materials. The results presented also show that rapid denitrification of nitrate in soil can be induced by incubating the soil under waterlogged conditions with organic materials such as glucose and that denitrification can be followed by total-N analyses if the organic material used to induce denitrification is not added in such excess that it also promotes significant fixation of atmospheric nitrogen. It seems likely that the fixation of

atmospheric nitrogen observed on incubation of waterlogged soils with nitrate and large amounts of glucose or straw did not commence until nitrate had practically disappeared since Delwiche & Wijler (1956) have shown that non-symbiotic nitrogen fixation in soil is suppressed by the presence of small concentrations of nitrate. The finding that ammonia accumulated in soil incubated under conditions which promoted fixation of atmospheric nitrogen is of interest since culture studies have shown that a considerable amount of the nitrogen fixed by *Clostridium pasteurianum* is excreted into the culture medium as ammonia (Zelitch, Rosenblum, Burris & Wilson, 1951).

The technique of incubating soil with nitrate and other materials in Kjeldahl flasks and determining loss of nitrogen by performing total-N analyses on the entire contents of the flasks eliminates the need to transfer or subsample the soil for analysis and permits accurate determinations of total-N changes in waterlogged soils. The data presented in Table 13 show that this technique gives highly consistent results when used to follow denitrification in soil and it has therefore been adopted in subsequent investigations on the factors affecting this process. The use of this technique for studies on denitrification in soil has the disadvantage that it requires the addition of nitrate at levels not normally encountered in the field, but this does not appear to be a serious defect since the experiments with nitrate at different levels showed that provided the supply of organic material was properly adjusted the same results were obtained whatever the level of nitrate added (Table 10 and Fig. 3). This finding has recently been confirmed by further experiments using labelled potassium nitrate which showed that the percentage loss of added nitrate-N on incubation of soil with nitrate and glucose under waterlogged conditions was practically the same whether the nitrate-N was added at the rate of 10 p.p.m. or 1000 p.p.m.

The finding that large quantities of nitrite were formed during the denitrification of nitrate in soil (Fig. 5) emphasizes the risks involved in the use of acid extractants for the determination of mineral nitrogen in studies on denitrification. Apart from the fact that nitrite is extensively decomposed and partially oxidized to nitrate during extraction from soil by acid reagents (Bremner & Shaw, 1955), it seems very likely that it is also lost through reaction with the organic matter in the soil since recent work (Bremner, 1957*b*) has shown that treatment of the humic acid fraction of soil organic matter with sodium nitrite and acetic acid leads to the production of a gas with the properties of nitrogen or nitrous oxide and to the fixation of nitrite-N by the humic acid fraction.

It is of interest in connexion with the work reported here to note that Hauck & Melsted (1956) have recently reported the results of attempts to recover all of the nitrogen originally present in soil treated with nitrate after incubation of the soil under conditions which induced denitrification. In their work they incubated soil with nitrate enriched with ^{15}N and determined the amounts of molecular nitrogen and nitrogen oxides produced (by analyses with the mass spectrometer and the infra-red spectrophotometer) and the change in the total-N content of the soil (by analysis using the salicylic acid modification of the Kjeldahl method). They found that by such analyses they were able to account for 89–100 % of the total nitrogen originally present in soil treated with nitrate after incubation of the soil in a closed system.

SUMMARY

1. Methods of investigating denitrification in soil are critically discussed with special reference to methods based on total-N analysis.

2. A modified Kjeldahl method of determining nitrogen which includes nitrate and nitrite and is applicable to waterlogged soil is described and the use of this method in studies on denitrification in soil is illustrated and discussed.

3. It is shown that rapid denitrification of nitrate in soil can be induced by incubating the soil under waterlogged conditions with organic materials such as glucose and that denitrification can be followed by total-N analyses if the organic material used to induce denitrification is not added in such excess that it also promotes significant fixation of atmospheric nitrogen.

4. The percentage of added nitrate-N lost by denitrification on incubation of waterlogged soils with different amounts of nitrate and sufficient glucose for denitrification was found to be the same whatever the level of application of nitrate.

5. Denitrification of nitrate in waterlogged soil containing glucose was found to be accompanied by a rapid but temporary accumulation of large quantities of nitrite and by the formation of smaller amounts of ammonia. Hydroxylamine could not be detected during denitrification, but it was found that this compound was rapidly decomposed in the soils examined by a process which appeared to be purely chemical.

6. It is shown that denitrification of nitrate in soil is a microbiological process and that the viability of the micro-organisms responsible for denitrification is not affected by air-drying and storage of the soil.

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